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(54) Title: TUMOR-DERIVED BIOLOGICAL ANTIGEN PRESENTING PARTICLES

(57) Abstract: Disclosed is a biological anti-tumor approach that delivers specific antigen stimulation in the presence of co-stimulatory signals to the immune system. Tumor cells are engineered such that processed antigens and co-stimulatory molecules are incorporated into virus-like particles capable of modulating immune responses without cellular entry and nucleic acid genomic host cell integration. The invention describes constructing antigen presenting particles that will themselves present tumor antigens in an immunogenic fashion to therapeutically activate anti-tumor immunity within a mammalian host.

Title Of The Invention:

Tumor-derived Biological Antigen Presenting Particles

Field Of The Invention:

This application is based on and claims priority of U.S. provisional application Ser. No. 60/499,758 filed September 3, 2003.

The present invention relates to the field of immune stimulation in mammalian cells, where biologically generated particles mimic antigen presenting cells by presenting to the host immune system tumor specific antigens in the presence of co-stimulatory molecules leading to immune awareness of *in vivo* tumor growth.

Background Of The Invention:

The single most important risk factor for cancer is age. Because the U.S. population is both growing and aging, the cancer burden in about 50 years from now by applying U.S. Census Bureau population projections to current cancer incidence rates will double. We can anticipate an increase from 1.3 million people in 2000 to 2.6 million people in 2050 diagnosed with cancer and the number of cancer patients age 85 and over is expected to increase four-fold in this same time period. Early diagnosis has led to more effective treatment, but these early detection methods are available for only a limited number of cancers. For many years, the treatment of cancer was primarily focused on surgery, chemotherapy, and radiation. But as researchers learn more about how the body fights disease on its own, therapies are being developed that harness the body's defense system in the fight against cancer. The body's immune defense system is a network of specialized cells. Therapies that use the immune system to fight cancer are biological therapies and the present patent application describes novel technology that can result in anti-tumor responses. The invention relates to a nanotechnology approach by creating particles (1 to 100 nanometers) with novel properties, properties that are normally found associated with cells. This invention application has tremendous potential to meet the present and future demand for therapeutic products for cancer biomedicine.

The enhancement of immune responses in the vaccine setting can be divided mechanistically into three categories: (i) Enhancement of the presentation of antigens to T cells, which implies an increase in peptide-MHC density at the site of activation of T cells; (ii) Enhancement of co-stimulation, which

accounts for the fact that T cells require extra co-stimulatory signals — either cell surface-bound molecules such as B7 or soluble molecules such as cytokines — in addition to engagement of the T cell receptor in order to become efficiently activated; (iii) Local elaboration of cytokines that attract and locally activate bone-marrow-derived antigen presenting cells that process and present tumor antigens to T cells. Most of these strategies are based on the engineering of tumor cells, requiring *in vitro* expansion of primary mammalian tumor explants. This, in addition to the potential hazards associated with cellular gene delivery approaches, present potential hazards in the genetically altered tumors-based enhanced immunogenicity approach. Other approaches may overcome the need to *in vivo* expansion of tumor tissues, but requires harvesting enough cells from the patient to produce the vaccine, limiting therapy to those where tumor cells could be collected through pheresia or surgical excision of the tumor mass. These cancers include leukemia, lymphoma, multiple myeloma, colon cancer, renal cancer, and ovarian cancer. The current technology as described above is problematic to cancer therapy for the mass population at risk; it is geared towards individual care, not to treat the large numbers of individuals that require treatment.

As an alternative to modifying the tumor cell, several approaches enhance antigen presenting cell (APC) function. Interest has been mainly focused on dendritic cells (DC), which represents the most potent professional APC. Dendritic cells have been shown to allow priming of naïve T cells and to initiate immune responses. They are unique APC that express a high level of MHC peptide complexes (signal 1) along with a collection of co-stimulatory signals (signal 2) — secreted cytokines and membrane-bound accessory molecules. These accessory molecules are representatives of the B7- and TNF-family of molecules. Since qualitative and quantitative aspects of T cell activation are determined by the co-stimulatory signals delivered by APC, much research has focused on dendritic cell associated molecules that regulate T cell biology. Within the B7-family of co-stimulatory molecules, B7-1 and B7-2, has been most widely studied in cancer vaccination, however new members are emerging — B7-DC, a molecule expressed exclusively on dendritic cells demonstrates potent synergistic T cell costimulatory effects in conjugation with B7-1 and B7-2.

The central role of antigen presenting cells in cancer vaccination has led to techniques for growth of dendritic cells (DC) in culture, resulting in *ex vivo* generated DC vaccines. However, technological problems face DC vaccine use. The prominently recognized issues are the timing of antigen(s) exposure

to the antigen presenting cells and the cytokines required to differentiate the antigen presenting cell towards the activated differentiation state. Not withstanding the costly labor-intensive limited availability therapeutic approach associated with DC cell-based therapy, the qualitative and quantitative nature of an immune response depends on the maturation and type of antigen presenting cell that processes and presents antigens to T cells. Although mature dendritic cells are more than 100 times more potent antigen presenting cells than other professional antigen presenting cells, such as B-cells and macrophages in activating naïve T cells *in vitro*, it is the early differentiating antigen presenting cell, not the mature dendritic cell, that posses the specialized antigen uptake and antigen processing machinery. Establishing the appropriate timing for antigen processing within *ex vivo* maturation of bone marrow antigen presenting cells have lead to a tripartite approach — lentiviral transduction of hematopoietic stem-progenitor cells, bone marrow transplant followed by infusion of mature post-thymic lymphocytes, and systemic infusion of Flt-3ligand/anti-CD40 — to increase the expression of antigens by dendritic cells *in vivo*. The potential opportunity to substitute dendritic cell *ex vivo* maturation / antigen presentation with pre-formed inactivated particles that are engineered biologically *in vitro* and implanted *in vivo* to directly interact with T cells to achieve the activated state for anti-tumor responses is the core premise of the present application.

The invention, as described in this application, is geared toward treatment strategies for the mass population at risk for cancer. Instead of cells, the biological particles/carrier approach, relies on particles; the expansion of particles exceeds the expansion of cells by greater than a million-fold since one modified cells can create at least 10E6 particles and if each particle is equivalent to each cell, there is a 10E6 economy of scale by producing particles to replace the current successes in cell-based modified tumor biology approach for cancer therapy.

Summary Of The Invention

This invention provides for the formation, production, and *in vivo* delivery of recombinant molecules for therapeutic purposes. The invention could contain one or more than one molecule or contain native cell surface components from a particular cell type. Molecules preferably include any amino acid moiety-containing molecule, but other molecules captured during the process covered by this invention could be envisioned. Formation of specific molecules could be engineered genetically by molecular biology techniques to be expressed on the surface of cells that alone or together with native

molecules on the said cells' surface, forms the essence of the invention. The formation and production of the invention involves the removal of cell surface membrane components as a consequence to the budding of particles from cells. The particles could be made of single or multiple components. Components are envisioned to be viral in origin, but could be induced by non-viral methods, or natural to the cell selected host. The invention is preferably for *in vivo* delivery, but could be used *in vitro* for induction or maintenance of cellular processes. Processes include, but not limited to, cellular signaling, cellular induction, cellular suppression and/or cellular attractant. *In vivo* delivery could be by intravenous injection, but other routes include but are not limited to oral, suppository, intra-muscular, inter-cranial, inter-peritoneal, or directly into mammalian organs, capillaries, ducts, or lymphoid system either alone or associated with biological or non-biological materials and/or devices. Inter-respiratory devices, cutaneous, and topical applications are also envisioned within this invention. In addition the application of the invention as aerosols, creams, puffers, or on surfaces, is included in this invention. Surfaces include, but not limited to, synthetic, non-synthetic, biological, or non-biological matrixes including autologous, allogeneic, and xenogeneic extracellular matrix materials. Therapeutic purposes encompass all procedures and/or processes that result in the improvement or intended improvement of the health and well being of an inflicted human or mammalian host.

This invention using a biological particle/carrier approach provides several advantages that would dramatically affect the outcome of vaccination. These parameters include:

(1) Ability to provide significant amount of MHC tumor peptide (signal 1) within an optimal immunologic context — co-stimulatory molecules for signal 2 — to provoke an effective anti-tumor immune response. In all preclinical models in which vaccine cell numbers were evaluated, increasing the number of vaccinating cells increase the potency of systemic immunity. For this purpose, the substitution of cells for particles may allow the delivery of more immunogenic material at the site of vaccination without requiring tumor cell expansion.

(2) Biological particles/carriers would allow a poly-epitopic presentation that would avoid tumor antigen and MHC variant loss following vaccination with a single epitope.

(3) Expression level of ligand / mediator containing particles could be engineered within established MHC-matched or possibility MHC-unmatched cell lines and released criteria established and tested *in vitro* to determine the ability of the preparation to induce immune responses. Established cell lines lend consistency between product batch production lots.

(4) Route of administration of the vaccine that is most effective at generating an effective anti-tumor immune response. As mentioned in the first parameter, cell dose is often limited in clinical testing related to the amount of material available to make the vaccine. For cell-based therapies, preclinical models are difficult to completely analyze routes of injection since the dermis of rodents is too thin to place large numbers of cells. This will not be the case for biological particles / carriers where particles are the size of viruses and are amendable to concentration of 100 to >1,000-fold, requiring tens to hundreds of microliters for dose delivery of >10E7 cell dose equivalence. However, the optimal route of administration may differ for different histological types of tumors, and therefore, additional routes of particle delivery are possible within a mammalian host. The biological particles / carriers technology lends itself to all types of delivery options, including aerosol and suppository administration.

(5) Ability of re-infused *ex vivo* generated dendritic cells to traffic intact to sites of antigen presentation — the spleen and draining lymph nodes — is limited, and those that do traffic appropriately are often cleared by host cytotoxic T-lymphocyte (CTL) activity. The carrier technology lends itself well to trafficking and evading clearance from the body while still maintaining immune stimulatory activity. Viruses are well known to navigate throughout the body without detection.

(6) Safety — the biological particles/carriers are not infectious, intended not to contain any genetic material. The viral-like components behave as a scaffold where appropriately configured protein molecular complexes are embedded.

Cancer can effect any organ in the human body, including breast, with 190,000 new cases reported each year; prostate with 180,000 cases diagnosed and treated annually with 32,000 annual deaths; lung with 175,000; colon with more than 94,000; head and neck with 70,000; gynecologic malignancies with more than 82,000; bladder with 55,000; pancreatic with 26,000; kidney with 31,000; brain with 17,900; sarcomas with 8,900 cases diagnosed on an annual basis, occurring in children and

adults. The present invention relates to treatments of these cancers, but not limited to these types. Current therapies include surgery, radiation therapy, chemotherapy, and bone marrow transplantation. New treatment approaches are needed to reduce costs. The biological particles/carriers technology could substantially improve and/or compliment current cancer therapies by using universal appropriately modified tumors as host cells to provide particles that will be the biopharmaceutical basis for treatment.

In addition, the present invention will simplify the manufacturing and production process for cancer vaccines. The technological innovation of this invention is that an established cell-mediated anti-tumor effect is reduced and simplified to a particle-mediated anti-tumor effect. The product is envisioned to be a lyophilized preparation stable at room temperature storage that has an economy-of-scale production and manufacturing advantage since each cell produces tens of millions of *gag* particles, each capable of inducing a T-cell response. The particles have no intrinsic activity. They are neither infectious nor self-replication competent, and are not intended to contain any nucleic acids. They are intended to mediate responses through cell surface interactions, triggering signal-transduction events upon engagement of cognate receptors on T cells *in vivo*, but could be envisioned as foreign bodies that could be taken up by other cell types for presentation to the host immune system. The commercial application is in the area of cancer where the product would greatly improve the availability of anti-tumor technology in a clinical setting.

In one aspect of the invention the host cell producing the biological particles/carriers will be an established tumor cell line. This cell line will have MHC molecules present on the cell's surface loaded with pre-processed antigens specific to that tumor. Co-stimulatory molecules could be engineered into the established tumor cell line by the introduction of specific cDNA by mechanical, physical, chemical, or viral means. Mechanical, physical, and chemical means include but not limited to electroporation, and/or lipid-mediated, polyethylene glycol, Sendai virus membrane fusion that bypasses the cellular membrane to gain access to the cellular chromatin structure where integration may or may not occur. Viral mediated delivery mechanisms include but not limited to murine leukemia virus (MuLV), adenovirus, adeno-associated virus (AAV), lentivirus, and canarypox vectors. In another embodiment of this aspect, the host cell for the biological particles/carriers could be primary cells derived from the tumor. In another embodiment of this aspect, the host cell for the biological particles/carriers could be a transformed cell line. In still another embodiment of this aspect, the host cell for the biological

particles/carriers could be a cell line not related to the tumor, but a universal cell line expressing specific (one or more) tumor antigens in the presence of co-stimulatory molecules. In all embodiments of this aspect, the host cell for the biological particles/carriers could be autologous, allogeneic, or xenogeneic with respect to the intended mammalian recipient of the therapy.

In all aspects of the invention the particle released from the host cells generating the biological particles/carriers is non-infectious. In one embodiment the biological particles/carriers production could be innate to or induced by the introduction into the host cell of viral or non-viral components by mechanical, chemical, and/or viral vector means. In another embodiment the biological particles/carriers production from the host cell could be due to the expression of one or more viral matrix proteins, for example, but not limited to, HIV-1 *gag* protein or the M1 matrix protein of the Influenza virus. In still another embodiment the biological particles/carriers released from the host cell could be an infectious viral particle that is later inactivated by various chemical means including, but not limited to nucleic acid crosslinking inactivation. In all embodiments the released particles could be harvested; concentrated by various methods, including, but not limited to polyethylene glycol; and lyophilized for long-term storage prior to therapeutic use *in vivo*.

The present invention describes an immune stimulation technology that has demonstrated the ability to stimulate T cells by incorporating over-expressed cell surface co-stimulatory proteins into either active or inactive viral particles and/or virus-like-particles. The process relies on the biological process of particle release to remove pieces of the cellular membrane while exiting a said host cell. Host cells are modified to release virus-like-particles or infectious virus particles that are subsequently inactivated together with recombinant co-stimulatory proteins displayed onto the surface of cells by standard molecular biological transfection and/or transduction techniques. The recombinant co-stimulatory proteins include, but not limited to CD40, CD40 ligand, CD30, CD30 ligand, 4-IBB receptor or ligand, CD27, FAS receptor or ligand, and TRAIL receptor or ligand. In fact, the recombinant protein or proteins that mediate the anti-tumor effect could be a cytokine or antibody that either directly or through accessory cells induce an immune response against the tumor. As a cytokine, the molecule could be, but not limited to an interleukin (IL-2, IL-12, IL-15, IL-23), a colony stimulatory factor (GM-CSF) or tumor necrosis factor (TNF-alpha). As an antibody, the molecular could be, but not limited to a T or B cell receptor component (anti-CD3 or anti-CD20); a co-stimulatory receptor (anti-CD28); or an

activation modulatory molecule (anti-CTLA4). In fact, the anti-tumor response could be due to apoptosis of immune reactive cells as could be the case in autoimmune diseases. As an example, but not limited to the disease—myasthenia gravis, particles containing an auto-reactive antigen in the presence of an apoptotic molecule could delete reactive immune cells. In all cases, the released particles contain the same over-expressed protein present on the host cells' surface and as such, serve as a novel delivery system for recombinant molecule signaling. In this way, single or multiple molecules are expressed with similar native structure to the naturally expressed human or mammalian protein.

The "capture" of a protein on the surface of a particle simplifies the process of synthesizing and purifying recombinant molecules and/or proteins to harvesting virus particles. Thus, *in vitro* recombinant protein systems can be simplified to purification of viral / non-viral / cellular particles or viral-like-particles using standard generic techniques. At the same time this technology insures proper orientation, conformation, and post-translational modifications of the synthesized protein, since the protein is made *de novo*.

In summary, the present invention describes the utility of a process to *in vivo* deliver immune modulator signals to a host immune system in the area of cancer biology, resulting in anti-tumor responses. The invention has applications to a wide range of tumors, including but not limited to direct presentation of specific tumor antigens in the context of MHC presentation with co-stimulatory signaling to T-lymphocytes. The invention mimics antigen-presenting cells with a similar efficiency as dendritic cells to present antigens to the immune system. The invention could also serve as a source of tumor antigen for uptake by host antigen-presenting cells, which in turn could present the tumor antigens to the host immune system. The invention provides a method to produce large amounts of material with efficacy similar to cell-based therapeutic approaches. The invention is a biological agent expressed as a particle containing one or more recombinant protein for *in vivo* use to induce, modify, and enhance immune cellular processes.

Brief Description Of The Drawings:

The invention is further described by the accompanying drawings and the description thereof herein, although neither is a limitation of the scope of the invention. Although the biological particle/carrier system has not been tested in the area of tumor biology, it has been tested and shown to

induce T cell proliferation. T-cell stimulation is a requirement for immune recognition and immune stimulation, resulting in anti-tumor responses.

Figure 1 is a schematic representation of constructing a virus-like particle producing tumor cell line. The figure illustrates the introduction by electroporation of plasmid vectors expressing a particle budding system. The particle budding system could be produced by a number of different mechanisms; as examples, the expression of the HIV-1 gag protein or the M1 matrix protein from the Influenza virus is shown. In this embodiment, a tumor cell line would be continuously processing cellular proteins and displaying the processed peptides in the context of class I and class II MHC molecules. Some of the processed cellular proteins would be tumor specific and in the presence of co-stimulatory molecules could induce immune reactions against that specific type of tumor. The introduction of the particle budding system into the tumor cell would release virus-like particles where the molecules present on the surface of the host tumor are incorporated into the particle. The end result of such modifications of the tumor cell is that the release particles can substitute for the tumor cells.

Figure 2 is a schematic representation of constructing a virus-like particle containing co-stimulatory molecules. The figure illustrates the introduction, by retroviral vectors, of particles containing co-stimulatory molecules. In this embodiment, B7 family co-stimulatory molecules are expressed on to the surface of tumor cells already expressing the particle budding system. As the particles are released from the tumor cells they would incorporate, in addition to MHC molecules containing processed peptides, co-stimulatory molecules. The introduction of co-stimulatory signaling molecules into the tumor cells along with MHC associated processed tumor antigens would impart onto the released particles antigen presenting capabilities. These capabilities could mimic cells present within the mammalian immune system to result in "dendritic-like" cells capable of anti-tumor responses.

Figure 3 identified the inability of biological particles / carriers released from viral infected host cell lines to induce T cell proliferation and activation. Two types of biological particles/carriers were made. The host cell used in the case of herpes simplex virus was, Lof(11-10), a fibroblastic line capable of infection by herpesviruses (HSV). The host cell used in the case of human immunodeficiency virus (HIV) was a chronic T cell line, A3.01, continuously expressing HIV particles. The viral-specific biological particles/carriers were collected from the supernatant of host cell cultures and inactivated with a UV-activated DNA crosslinker. The preparations were non-infectious by the lack of p24 release from

exposed CD4+ T cells (HIV) or lack of cytopathic cell lyses in susceptible fibroblasts (HSV-2). The experiment shown used human peripheral blood mononuclear cells (hPBMCs) from 5 different healthy donors. The hPBMCs were exposed to the biological particle / carrier preparations at the start of culture, time points were taken (only 1 shown), and proliferation assays perform using AlamarBlue™. The biological particle/carrier preparations were derived from either unmodified host cells. For the three hPBMC donor cells tested, the degree of T-cell proliferation upon exposure to biological particle/carrier preparations derived from unmodified cells was equal to that observed in untreated hPBMCs and not to that observed in PHA-stimulated cultures, demonstrating that the released native particles are not intrinsically immune stimulatory.

Figure 4 identified the ability of biological particles / carriers released from co-stimulatory modified viral infected host cell lines to induce T cell proliferation and activation. The same 2 types of biological particles/carriers made in Figure 3 were used here. The hPBMCs were exposed to biological particle / carrier preparations derived from either unmodified or co-stimulatory modified host cells. For the three hPBMC donor cells tested, the degree of T-cell proliferation upon exposure to biological particles/carriers (either HSV- or HIV-based) derived from co-stimulatory modified cells were stimulatory. Whereas the hPBMC cultures exposed to biological particles/carriers from unmodified host cells were not, similar to that shown in Figure 3. Co-incubation experiments with host cells and hPBMCs showed that only the B7-modified host cells stimulated T-cells. The data supports the notion that released particles retain the stimulatory activity of the host cells and the addition of B7 to the host cells resulted in particles that could stimulate T cell proliferation.

Detailed Description Of The Preferred Embodiments

The present invention relates to the use of particles to capture and incorporate surface molecules that are displayed naturally and/or purposely expressed on the surface of host cells by recombinant molecular biologic techniques. The naturally displayed molecules could be tumor–derived processed antigens associated with MHC molecules or molecules that assist in presenting processed antigens to the immune system. The assisting molecules could be from the class of molecules known as co-stimulatory molecules that consist of surface expressed molecules (B7 family members, members of the TNF family, and/or other immunoglobulin family members—ICAM and VCAM), cytokines (interleukins and